

EXINE REDUCTION IN UNDERWATER FLOWERING
CALLITRICHE (CALLITRICHACEAE):
IMPLICATIONS FOR THE EVOLUTION
OF HYPOHYDROPHILY

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ABSTRACT

Both aerial and underwater pollination systems (hypohydrophily) occur in *Callitriche* (Callitrichaceae), and as such the genus can serve as a model system in which to probe questions concerning the evolution of hypohydrophily from aerial pollination systems. Evidence from scanning and transmission electron microscopy reveals strikingly different exine thickness in the pollen of four aerially flowering species (*C. heterophylla* var. *bolanderi*, *C. marginata*, *C. peploides*, and *C. stagnalis*), which have a distinct sexine layer (0.55–1.1 μm thick), in comparison with that of the hypohydrophilous *C. hermaphroditica*. The exine of the latter species is rudimentary (<0.1 μm thick) in pollen of the annual form of the species, and virtually absent in the perennial form. The degree of exine reduction in pollen of *C. hermaphroditica* relative to that of the aerially flowering species contrasts with the otherwise general morphological similarity observed among the flowers of the respective species.

Key Words: *Callitriche*, underwater pollination, pollen ultrastructure

INTRODUCTION

Although considerable insight has been gained regarding pollen form and function in angiosperms (e.g., Blackmore and Ferguson, 1986 and refs. therein) a general consensus remains elusive concerning possible relationships between pollen morphology and pollination systems. Several studies have shown positive correlations between pollen structure and the nature of the pollen vector (e.g., anemophilous compositae, Bolick, 1990; papilionoid legumes, Ferguson and Skvarla, 1982; Araceae, Grayum, 1986; Cambombaceae, Osborn, Taylor, and Schneider, 1991), whereas others report a lack of such associations (e.g., Polemoniaceae, Taylor and Levin, 1975; various families, Lee, 1978; Zingiberales, Kress, 1986). Factors that are indirectly related to pollination may also play a role in influencing the evolution of pollen structure, especially pollen–stigma interactions (see Kress, 1986).

In contrast, a striking correlation exists between pollen structure and pollination system in species that exhibit underwater cross-pollination systems (hypohydrophily). Reduction of exine thickness, or the lack of exine altogether, is strongly correlated with

hypohydrophily (Cox, 1988; Philbrick, 1988; Philbrick and Anderson, 1992). For instance, pollen of hypohydrophilous species from often distantly related groups have rudimentary exines, including *Ceratophyllum demersum* L. (Ceratophyllaceae: Les, 1988), *Enhalis acoroides* (L.) Royle and *Thalassia hemprichii* (Ehrenb.) Aschers. (Hydrocharitaceae: Pettitt, 1980, 1981), while an exine component of the pollen wall is absent in *Thalassodendron ciliatum* (Forsk.) den Hartog (Hydrocharitaceae; Pettitt, 1980), and species of *Najas* (Najadaceae: Blackmore, McConchie, and Knox, 1987) and *Amphibolis* (Cymodoceaceae: Pettitt, Ducker, and Knox, 1978). Indeed, exine reduction is one of the few morphological features that are unique to hypohydrophilous pollination systems.

Few comparative studies have been conducted at the infrageneric level between aerially flowering and hypohydrophilous species. Clearly the major hindrance to such comparisons is the fact that genera that have both pollination types are exceedingly rare. As a consequence, our understanding of the nature of the changes that take place in pollen structure, indeed in all floral structure, during the evolution of hypohydrophily are based on comparisons at the generic level and above. Yet, comparisons at these higher taxonomic/phylogenetic levels are of limited value for understanding features that are as evolutionarily dynamic as pollination systems. *Callitriche* is an ideal genus for such comparisons because both aerial and hypohydrophilous pollination systems occur.

Callitrichaceae is a widespread monogeneric (*Callitriche*) family of ca. 50 species. Species of *Callitriche* exhibit one of three growth habits: terrestrial, amphibious, or aquatic (Philbrick and Anderson, 1992 and refs. therein). Terrestrial species occur in seasonally wet areas while plants of amphibious species can grow submersed, with a floating rosette of leaves, or as the land-form on moist ground. Aquatic species grow obligately submersed.

Callitriche is the only genus in which both aerial floral biologies and hypohydrophily have been documented (Philbrick and Anderson, 1992; Philbrick, 1993). The majority of species of *Callitriche* flower aerially; anemophily seems to be the principal pollination system. *Callitriche truncata* Gussone and *C. hermaphroditica* L. show obligately submersed flowering. Philbrick (1993) has employed paternity exclusion analysis using random amplified polymorphic DNA markers to document hypohydrophily in *C. hermaphroditica*. The pollination system of *C. truncata* is poorly known.

The high degree of floral structural divergence between aerial flowering and hypohydrophilous angiosperms is well known (e.g., Arber, 1920; Dahlgren and Rasmussen, 1983; Philbrick, 1991; Sculthorpe, 1967; Tomlinson, 1982). A significant component of this divergence entails loss or reduction of parts (e.g., perianth, number of stamens) and reduction in flower size. Consequently, one would predict that a similar degree of divergence would occur between aerially flowering and hypohydrophilous species of *Callitriche*. However, this is not the case. The overall floral morphology of species of *Callitriche* is uniform (cf., Philbrick and Anderson, 1992; Schotsman, 1982, 1985). The only qualitative difference in reproductive structures of aerial and submerged flowering species is the presence (aerial flowering species) or absence (submerged flowering species) of endothelial thickenings in the anthers (Schotsman, 1982; Philbrick, unpubl.).

Although several studies of pollen morphology of *Callitriche* have been published based on data obtained using light and scanning electron microscopy (SEM) (e.g., Diez, Talavera, and Garcia-Murillo, 1988; Martinsson, 1993; Moar, 1960; Moore and Webb, 1978) the diversity of pollen ultrastructural features that occur in the genus are not well understood. Only two investigations have provided structural information on *Callitriche* pollen based on transmission electron microscopy (Martinsson, 1993; Osborn and Philbrick, 1994).

Herein we address the question, how does exine thickness in the hypohydrophilous *Callitriche hermaphroditica* compare with that of aerial flowering species? This question is addressed by comparative study of exine thickness of four aerially flowering species of *Callitriche* and the hypohydrophilous *C. hermaphroditica*.

MATERIALS AND METHODS

Species Studied

Five species were studied: *C. hermaphroditica* L., *C. heterophylla* var. *bolanderi* (Hegelm.) Fassett, *C. marginata* Torrey, *C. peploides* Nutt., *C. stagnalis* Scop. Voucher specimens are listed in Appendix I. Four of the five species studied (except *C. hermaphroditica*) are aerially pollinated, and are presumed to be anemophilous although little experimental evidence confirms this.

Callitriche hermaphroditica is hypohydrophilous (Philbrick, 1993). One species (*C. heterophylla* var. *bolanderi*) was also selected that exhibits internal geitonogamy, an unusual form of self-fertilization (Philbrick, 1984; Philbrick and Anderson, 1992).

The species selected represent all three growth habits that occur in the genus. *Callitriche heterophylla* var. *bolanderi*, *C. marginata*, and *C. stagnalis* are amphibious. *Callitriche peploides* is terrestrial and *C. hermaphroditica* is aquatic. Moreover, these species represent the three main phylogenetic lineages in the genus (Philbrick and Jansen, 1991).

Electron Microscopy

Entire plants were fixed in formalin : glacial acetic acid : 95% ethanol (FAA; 0.5:0.5:9). For SEM, pollen of all species except *C. hermaphroditica* was acetolyzed and prepared as outlined in Bogle and Philbrick (1980). Grains of *C. hermaphroditica*, which collapse even after mild acetolysis, were critical point dried, dehydrated in a graded ethanol series, and placed onto aluminum stubs. Grains were examined on scanning electron microscopes at the University of Connecticut and the Rancho Santa Ana Botanic Garden.

For transmission electron microscopy (TEM), mature anthers were excised from preserved plants, post-fixed in 1% osmium tetroxide, buffered in sodium cacodylate to a pH of 6.5, for 2 h and washed with buffer. Entire anthers were placed onto cellulose filters and then coated on both sides with agar. Agar embedded filters were subsequently dehydrated in a graded ethanol series, transferred to several changes of 100% acetone (to solubilize the filters), gradually infiltrated with Spurr low viscosity epoxy resin, and embedded. Ultrathin sections were cut with a diamond knife, collected on copper 1 × 2 mm slot grids, and dried onto formvar support films following the techniques of Rowley and Moran (1975). Grids were stained with 1% potassium permanganate (1–5 min.), 1% uranyl acetate (8–15 min.), and lead citrate (5–10 min.; Venable and Coggeshall, 1965), and examined using a Zeiss EM-10 transmission electron microscopy at 60–80 kV.

RESULTS

Both intine and exine sporoderm components of the grains were present, at least in part, in the pollen of all five species examined.

Not surprisingly, given that grains were fixed in F.A.A., the degree to which the intine was preserved in the various species differed. This limited interpretation regarding intine structure, but did not adversely affect interpretation of exine thickness, the main focus of the study. In general, the intine of *C. hermaphroditica* was well preserved while in the other four species it was not. The sporoderm fine structure of nonapertural wall regions, with particular emphasis on the layers, is presented below for each species.

C. heterophylla var. *bolanderi*

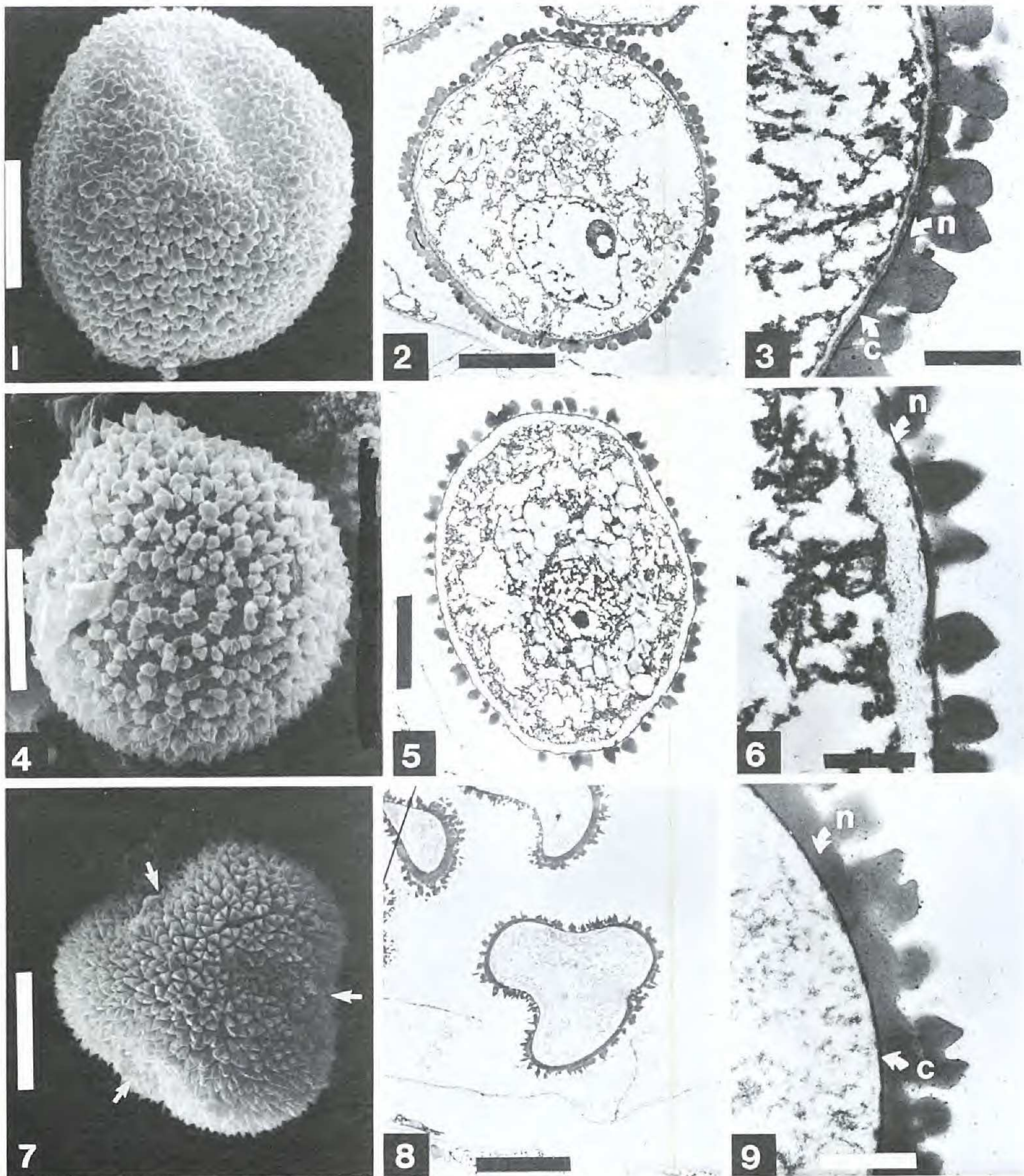
The grains of *Callitriche heterophylla* var. *bolanderi* (Figures 1–3) had a distinct exine component that ranged in thickness from 0.75–1.00 μm . The exine was intectate, and two-layered (sexine, nexine; cf. Erdtman, 1952), with the layers separated by a dark-staining commissural line (Figure 3). The sexine was composed of variably sized gemmae that formed an anastomosing to somewhat reticulate pattern (Figure 1). The gemmae were laterally fused at their bases, and in association with smaller granular elements formed a thin, somewhat irregular nexine (Figure 3). The nexine comprised about 20% of the total thickness of the overall exine. Below the commissure, relatively small granular elements of the nexine protruded into the underlying intine (Figure 3).

C. marginata

Pollen of *C. marginata* had a well-defined, two-layered, intectate exine that ranged from 0.55–1.17 μm in thickness (Figures 4–6). The sexine consisted of distinct elements ranging from gemmae to echinae that were fused laterally into a semi-reticulate pattern (Figure 4). A thin, somewhat continuous granular nexine was also present (Figure 6), and comprised approximately 10% of the total thickness of the exine. Larger granule units of the nexine were present below the more continuous layer, and penetrated into the underlying intine (Figure 6). A commissural line was indistinct.

C. peploides

Pollen of *C. peploides* had a distinct, intectate exine that ranged from 0.70–0.95 μm thick (Figures 7–9). The exine of this species



Figures 1–9. Scanning (Figures 1, 4, 7) and transmission (Figures 2, 3, 5, 6, 8, 9) electron micrographs of *Callitriche* pollen. Figures 1–3; *C. heterophylla* var. *bolanderi*. 1. View showing interapertural region, aperture is evident as an elongate depression in the upper right region of the grain. Bar = 5 μm . 2. Transverse section of entire grain. Bar = 5 μm . 3. Detail of nonapertural exine in transverse section; note the nexine (n) and commissural line (c). Bar = 1 μm . Figures 4–6; *C. marginata*. 4. View showing the interapertural region. Bar = 5 μm . 5. Transverse section of entire grain. Bar = 5 μm . 6. Detail of nonapertural exine in transverse section; note the nexine (n) relative to the thickness of the sculptured sexine. Bar = 1 μm . Figures 7–9; *C. peploides*. 7. Polar view and the location of the three apertures (arrows); note the organization of the sculptural elements into polygonal shaped clusters. Bar = 5 μm . 8. Transverse section of entire grain showing the location of apertures, denoted by the concave areas; note that apertural regions also have sculptured sexine elements, but a thinner nexine in comparison with nonapertural areas. Bar = 7 μm . 9. Detail of nonapertural exine in transverse section; note the relative thickness of the nexine (n) versus the sculptured sexine, and the commissural line (c). Bar = 1 μm .

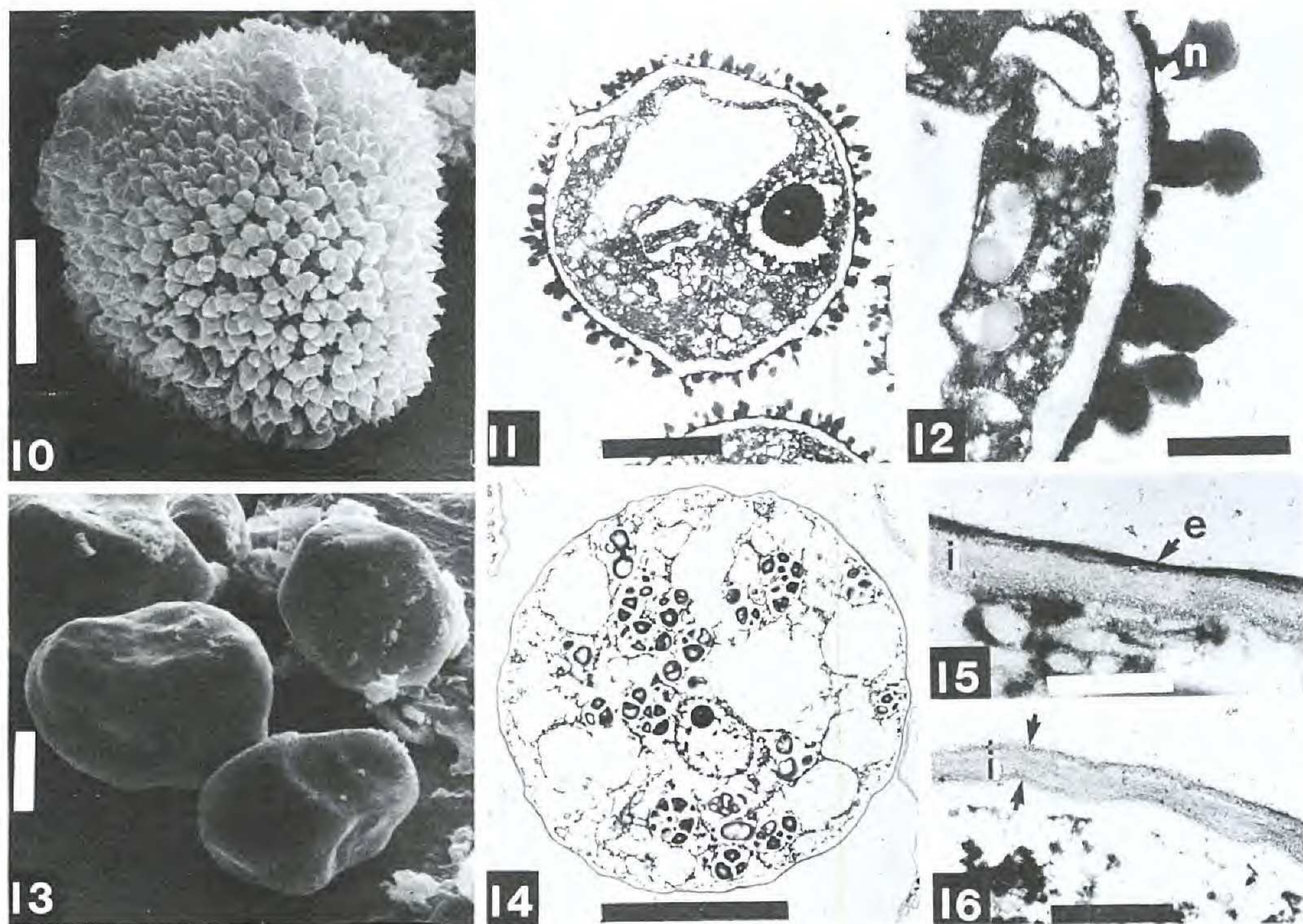
was also two-layered, with the sexine being composed of irregularly shaped verrucae and echinae. These outer sculptural elements were laterally fused at their bases and arranged in groups of 4–8 into polygonal shaped clusters (Figure 7). The sculptural elements were borne upon a relatively thick, homogeneous to granular nexine (Figure 9). The nexine of *C. peploides* was the thickest of the five species examined and accounted for about 30% of the total exine thickness. A dark commissure was present near the base of the nexine, and was in turn underlaid by a thin layer, that was somewhat irregular in thickness, consisting of minute granules (Figure 9).

C. stagnalis

Callitriche stagnalis had pollen with a well-defined intectate exine that ranged from 0.50–0.95 μm in thickness (Figures 10–12). Distinct clavate and echinate elements characterized the sexine along with a thin, granular nexine (Figure 12). The nexine comprised approximately 17% of the total exine thickness. The structural elements were variably fused and arranged into a somewhat reticulate pattern (Figure 10). A dark-staining commissural line was present within the nexine, and underlaid by a layer of irregular relatively small granules (Figure 12).

C. hermaphroditica

Callitriche hermaphroditica had the most distinctive pollen wall structure of the species examined (Figures 13–16). Grains had a well-developed intine ranging from 0.25–0.45 μm in thickness (Figures 15 and 16). In contrast to the other species examined, the exine of *C. hermaphroditica* is best described as rudimentary. However, ultrastructural variability was evident, even among grains within the same anther. In plants of the perennial growth form (#2267), an exine wall component was virtually absent (Figure 16), while in the annual form (#2030) the exine formed a thin, homogeneous to granular band that was generally less than 0.1 μm in thickness (Figure 15). In other grains from the annual growth form, the exine element was manifested as small, irregularly shaped granules that ranged in size from 0.05–0.16 μm (not shown). The poorly developed exine in *C. hermaphroditica* is likely the cause for the irregular shape and smooth external appearance of the grains (Figure 13).



Figures 10–16. Scanning (Figures 10, 13) and transmission (Figures 11, 12, 14–16) electron micrographs of *Callitriche* pollen. Figures 10–12; *C. stagnalis*. **10.** View showing the interapertural region. Bar = 5 μm . **11.** Transverse section of an entire grain. Bar = 5 μm . **12.** Detail of transverse section of exine; note the thin nexine (n) and the thicker sculptured sexine. Bar = 1 μm . Figures 13–16; *C. hermaphroditica* (perennial form [#2267]: Figures 14 and 16; annual form [#2030]: Figures 13 and 15). **13.** View showing several nonacetolyzed grains; note the irregular shape. Bar = 10 μm . **14.** Transverse section of an entire grain; note the absence of a well-defined and darkly stained outer sporoderm layer (exine). Bar = 5 μm . **15.** Detail of transverse section of sporoderm; note the relatively thin, rudimentary exine (e) and the thicker underlying intine (i). Bar = 0.6 μm . **16.** Detail of transverse section of sporoderm showing the virtual absence of an exine component and relatively thick intine (i, between arrows). Bar = 0.6 μm .

DISCUSSION

This study confirms exineless pollen in *Callitriche*. The general pattern of a well developed exine in aerially flowering species and the virtual lack of an exine in the hypohydrophilous *Callitriche hermaphroditica* is demonstrated. However, exine thickness differed in annual (rudimentary) and perennial (absent) forms of this species. Martinsson (1993) reported reduction, but not absence, in numerous accessions of this species from Sweden. The significance of this infraspecific variation in exine thickness is not known, but may reflect the recent origin of hypohydrophily in *C. hermaphroditica* (see below, and Osborn and Philbrick, 1994).

There is also an apparent association between the growth habit (terrestrial versus amphibious) and the relative thickness of the basal layer. Pollen of the terrestrial species (*Callitriche peploides*) had a thicker nexine (30% of total exine) than any of the three amphibious species (10–20% of total exine). However, only a single terrestrial species was examined. Additional terrestrial species need to be examined before the significance of this apparent difference can be interpreted.

Although the lack (or extreme reduction) of an exine is closely correlated with hypohydrophily, the adaptive significance of this association is not understood. It is perhaps intuitive to predict that the loss of exine is a result of its release from selection pressures that maintain it. Yet, it is not known whether the exine is actually selected against during the evolution of hypohydrophily or is simply lost due to genetic drift. In general, the harmomegathic nature of the pollen exine is associated with controlling water relations of the grain during dispersal (desiccation) and hydration on the stigma (Heslop-Harrison, 1971). It is not clear whether this alone would translate into the loss of exine when such extremes in water potential are absent during hypohydrophilous pollination. On the other hand, there may be direct selective advantages associated with the lack of exine. Perhaps the pollen delivery system itself exerts selective pressures (e.g., Cox, 1988). Relationships among exine reduction and factors such as pollen-stigma interaction, emergence of the pollen tube, and pollen dispersal remain to be clarified in hypohydrophilous plants. It is also important to note that there are several examples of aerially flowering groups that have extremely reduced exines. The adaptive significance of exine reduction in these groups is also equivocal, but does not seem to be closely related to pollination systems (Kress, 1986).

Hypohydrophily is clearly a derived pollination system in angiosperms; the aerial floral biology is abandoned in favor of the release and capture of wet, water-borne pollen. In a functional sense hypohydrophily represents one of the most unique (divergent) forms of pollination in angiosperms. Because of the close relationship between floral form and pollination system function (e.g., Faegri and van der Pijl, 1979) it is intuitive to predict that the floral structure of hypohydrophilous species would reflect the functional divergence of the pollination system (i.e., be markedly different than related aerially flowering species). However, this

does not seem to be the case in *Callitriche* where relatively little modification in overall floral morphology is apparent. The degree of exine reduction in *C. hermaphroditica* contrasts markedly with the morphological similarity that otherwise characterizes the flowers, which may indicate that the strongest selective pressures that have operated during the evolution of hypohydrophily in *Callitriche* occur on pollen, not other floral characters.

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APPENDIX I

Collection information of specimens examined during this study. All voucher specimens are located in the herbarium of Western Connecticut State University. *C. heterophylla* var. *bolanderi*: WASHINGTON, Thurston Co., 22 June 1987, *Philbrick* 2098. *C. marginata*: CALIFORNIA, San Diego Co., 6 January, 1986, *Philbrick* 1597. *C. peploides*: ALABAMA, Conecuh Co., 14 March, 1988, *Philbrick and Haynes* 2135. *C. stagnalis*: WASHINGTON, Gray's Harbor Co., 20 June, 1987, *Philbrick* 2096. *C. hermaphroditica*: annual form—CALIFORNIA, Tuolumne Co., 31 May, 1987, *Philbrick* 2030; perennial form—CALIFORNIA, Shasta Co., 12 June, 1990, *Philbrick* 2267.